Molecular Biology
BT201

Prepared and Presented by

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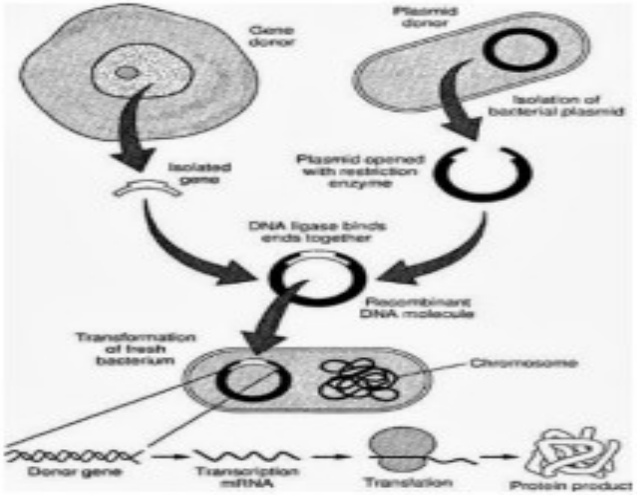
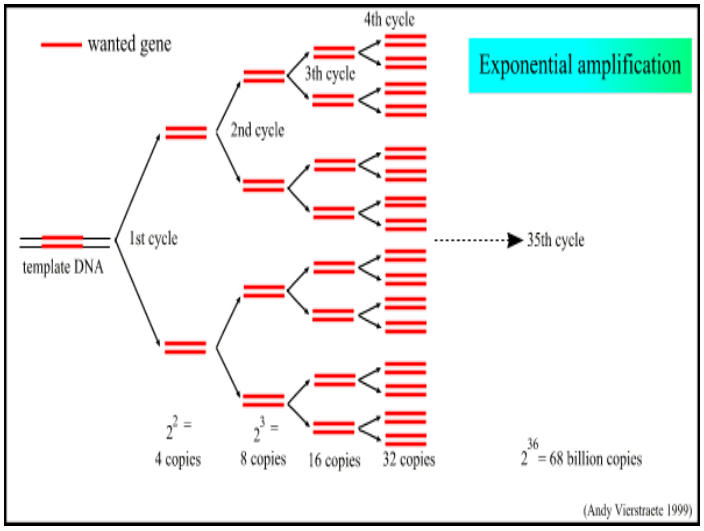
Amplifying DNA segments

Cloning (*in vivo*)



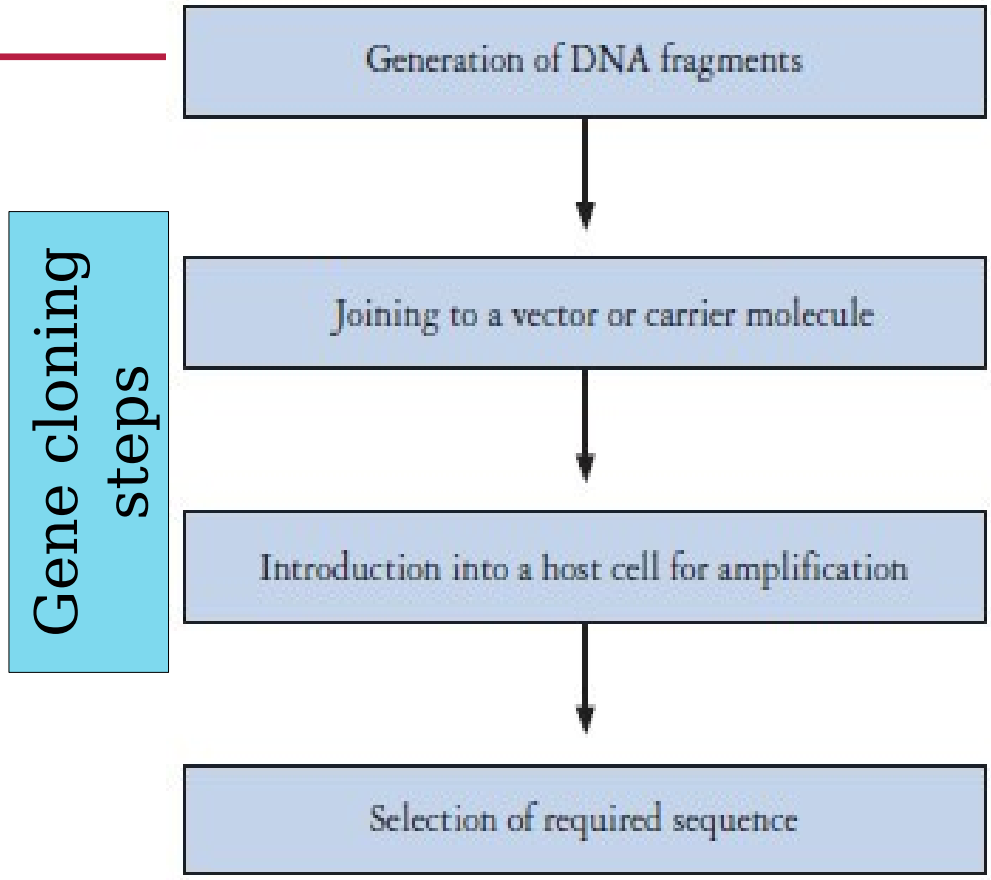
PCR (*in vitro*)



What is gene (DNA) cloning?

- DNA cloning is to make many copies of the same DNA sequence (gene, a part of gene, or non-coding DNA) using a living organism as the replication machine.

- This is referred to as ***recombinant DNA technology.***


Gene cloning steps
Generation of DNA fragments
Joining to a vector or carrier molecule
Introduction into a host cell for amplification
Selection of required sequence

What is the vector??

- **Is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed.**

- **A vector containing foreign DNA is termed recombinant DNA.**

DNA cloning requires

1- DNA we want to clone.

2- A vector: an agent that can carry DNA fragment into a host cell. There are two types of vectors:

A- Cloning vector: used to reproduce DNA

B- Expression vector: used to express a specific gene into a protein.

- ***Examples of cloning vectors:***

Plasmid: average of insert size is 0.1-10 kb.

Lambda phage: 8- 20 kb

Cosmid: 30-50 kb

BAC (bacterial artificial chromosome): 75-300 kb

YAC (yeast artificial chromosome: 400 kb

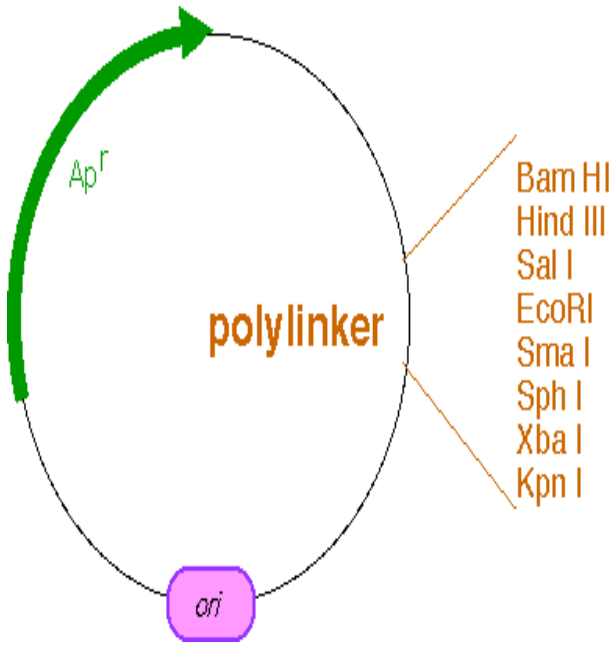
3- Host cell (**competent** bacteria)

characteristics of cloning vectors:

1. They contain an **origin of replication** that allows them to replicate independently of the bacterial chromosome.

2. They contain a **drug resistance gene (or selectable marker)** that allows cells that contain the vector (and any attached DNA) to be readily identified.

3. Includes multiple cloning sites, **"MCS"** or **polylinker**.



4-Screening marker: screening successful clones (i.e. the foreign DNA has been inserted into the vector) (ex., Lac Z gene).

5- **Elements for expression and Protein tags**:

(Expression vectors)

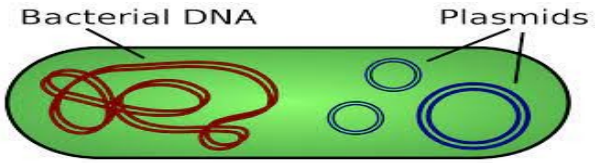
i- such as promoter, ribosomal binding site, start codon, termination codon, etc, for the expression of the target gene.

ii- used to separate and purify the expressed protein of interest from the great majority of proteins of the host cell.

Two kinds of DNA molecule that satisfy these criteria : **plasmids** and **bacteriophages**.

A-Plasmid

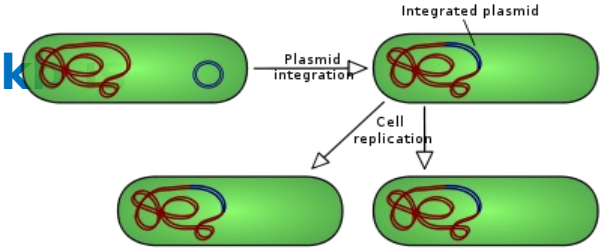
1- are extra **circular molecules of DNA** that have an independent existence in the bacterial cell



2- Several copies 1- several 100/ cell

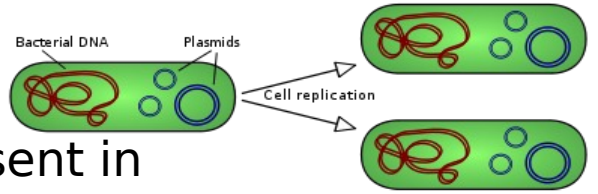
3- Size 1 Kb - > 250 Kb (**Plasmid less than 10 k**

desirable for a cloning vector).



4- Replication:

i- Stringent plasmids :



replicate once per cell cycle of bacteria thus present in low copy number (1 or 2) per cell.

ii- Relaxed plasmids:

replicate independently of the bacterial DNA thus present in high copy number (50 or more) per cell.

(A) Smaller plasmids make use of the host cell's own DNA replicative enzymes in order to make copies of themselves.

(B) larger plasmids carry genes that code for special enzymes that are specific for their own replication.

(C) episomal plasmid are able to replicate by inserting themselves into the bacterial chromosome. This is known as chromosomal integration (episomal integration).

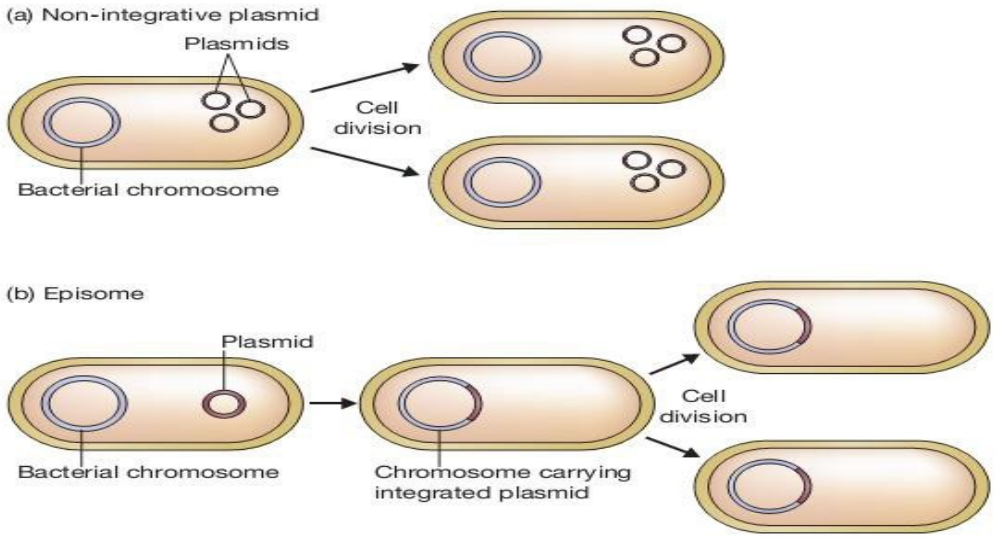
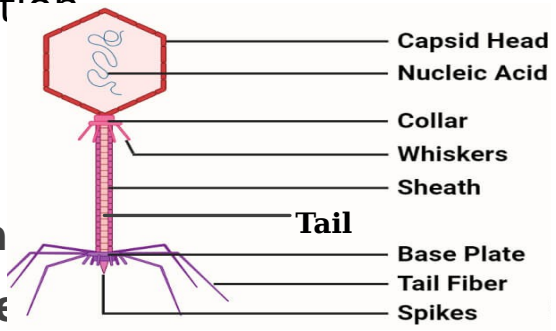


Figure 2.3
Replication strategies for (a) a non-integrative plasmid, and (b) an episome.

B-Bacteriophage (phages)

1- Viruses that are dependent on bacteria for their propagation



2- consisting merely of a **DNA (or occasionally RNA) molecule** carrying several genes and **surrounded by a coat or capsid, which is made up of protein molecule**

3- Bacteriophage advantages over plasmids:

a. DNA insert size up to 25 kb.

b. phages actively infect host bacteria; thus, their efficiency is higher than bacterial transformation.

c. Formation of plaques from the lysis of bacterial cell infection.

4- One of the major drawbacks of phage vectors is that the capsid limits the size of inserted DNA.

Basic steps of cloning foreign DNA using a plasmid vector

1- DNA or RNA extraction

2- Isolation of the desired insert to be cloned

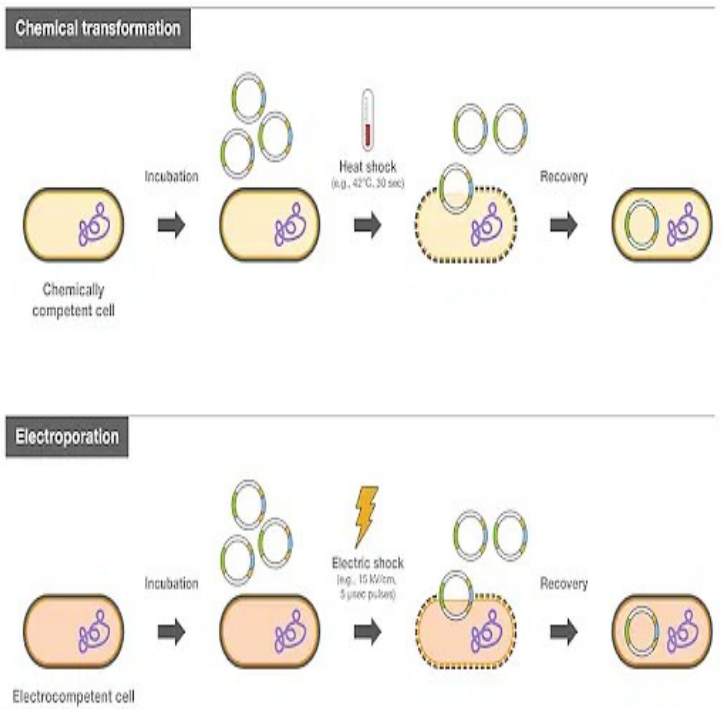
3- Choosing a suitable vector

4- DNA recombination:

- Plasmid vector and the foreign DNA are cleaved using the same restriction enzyme, and sticky ends are created.
- Sticky ends of plasmid vector and foreign DNA fragment are ligated together, forming “recombinant DNA”

5-Preparation of Competent cells

- **Competent cells**: microbial cells that can take up foreign DNA from their surroundings through a process called transformation.
- Preparation of competent E. coli cells:
  a. Chemically competent cells ($CaCl_2$ / heat shock method): increasing permeabilization of the cell membrane, allowing plasmid entry.
  b. Electroporation competent cells: electrical pulses create temporary pores in the cell membrane, which allows recombinant DNA entrance into the cell.



6- Transformation:

- Recombinant DNA is introduced to the host cells that have been made transiently permeable to DNA (ie, bacterial cell like *E. coli*).

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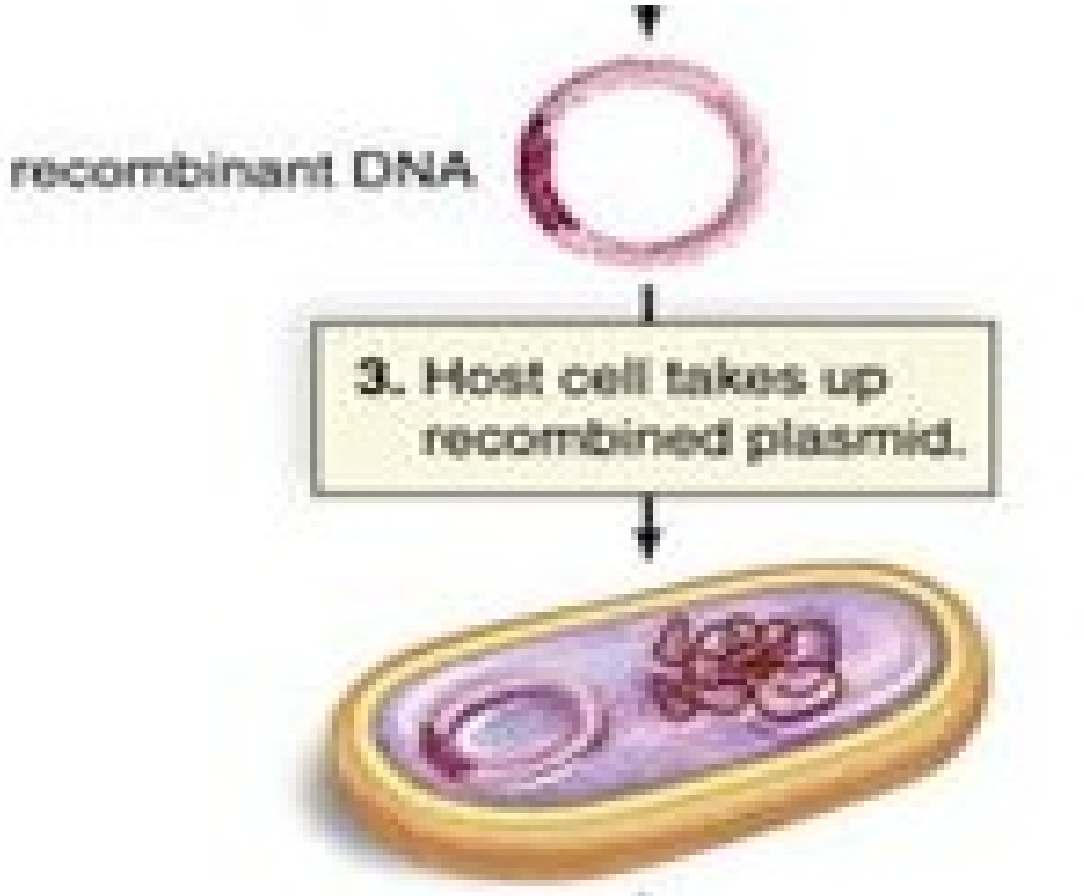
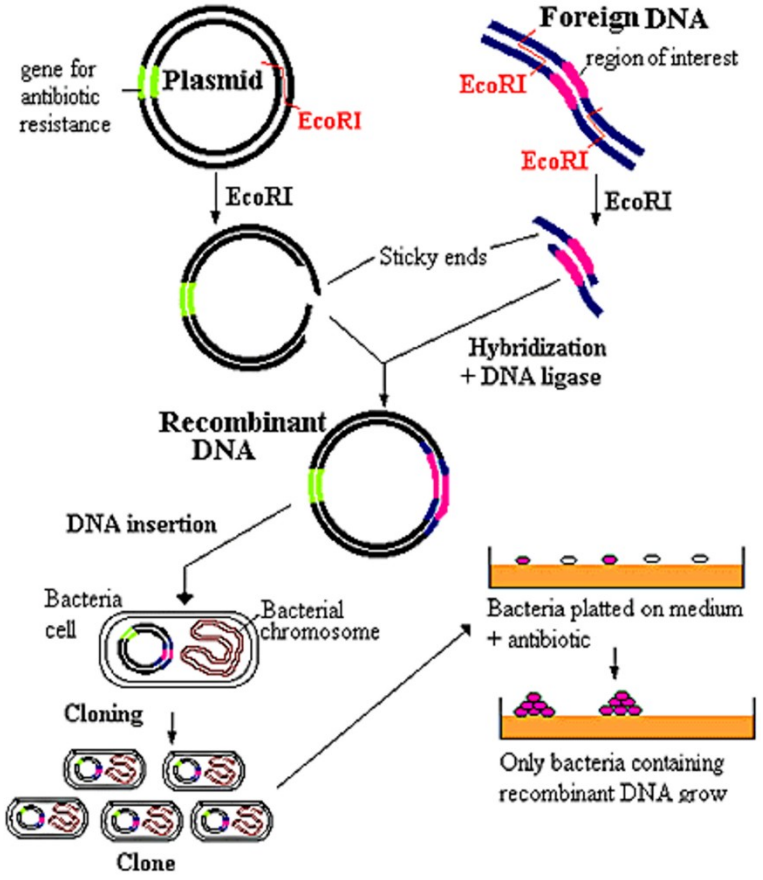


7- Selective amplification

- the ability to select clones of the desired gene, because the ligation mixture contains:
  - Desired recombinant molecule.
  - Unligated DNA fragments.
  - Vector molecules that have **re-circularized,** without new DNA being inserted (self-ligated vector).

- Selecting transformed bacterial cells (i.e., carrying native vector or recombinant vector).
- Only transformed cells express a certain antibiotic protein, allowing them to survive in the media with the selected antibiotic

- ex. Adding antibiotics to the media only allows the transformed bacteria to succeed in growing **in the media**.

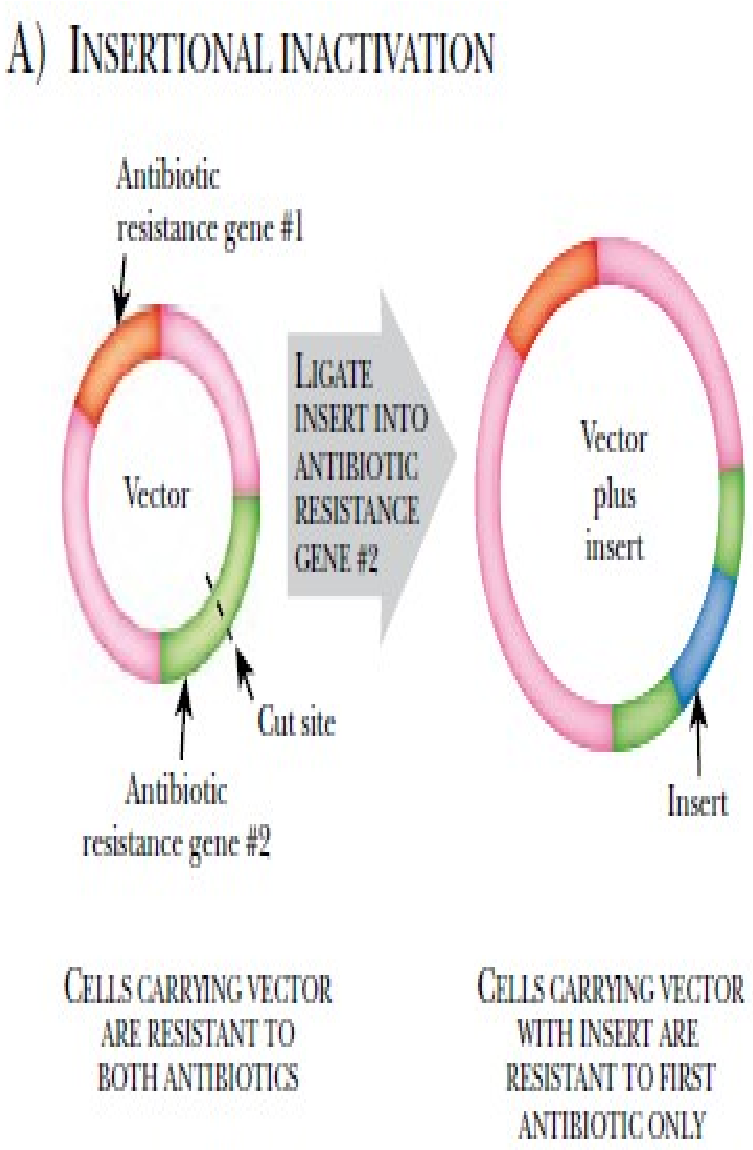


8-Screening

- To distinguish between recombinant and non-recombinant vectors

A-Insertional inactivation

- the vector has two different antibiotic resistance genes (eg. Ampicillin and tetracycline).
- One antibiotic resistance gene will be used for selection.
- While the other antibiotic resistance one will be used for the insertion of foreign DNA, and thus inactivate it.


A) INSERTIONAL INACTIVATION
Antibiotic resistance gene #1
Vector
Cut site
Antibiotic resistance gene #2
LIGATE INSERT INTO ANTIBIOTIC RESISTANCE GENE #2
Vector plus insert
Insert
CELLS CARRYING VECTOR ARE RESISTANT TO BOTH ANTIBIOTICS
CELLS CARRYING VECTOR WITH INSERT ARE RESISTANT TO FIRST ANTIBIOTIC ONLY

- The colonies are **replica plated**, using a sterile **velvet pad,** onto plates containing tetracycline in their medium.

- Colonies that **grow on ampicillin** but not on tetracycline must contain **plasmids with inserts (recombinant plasmid).**

- **Compare the replica plated plate to the master plate** to recognize the colonies with inserts and to recover them from the ampicillin plate for further growth.

Replica plating

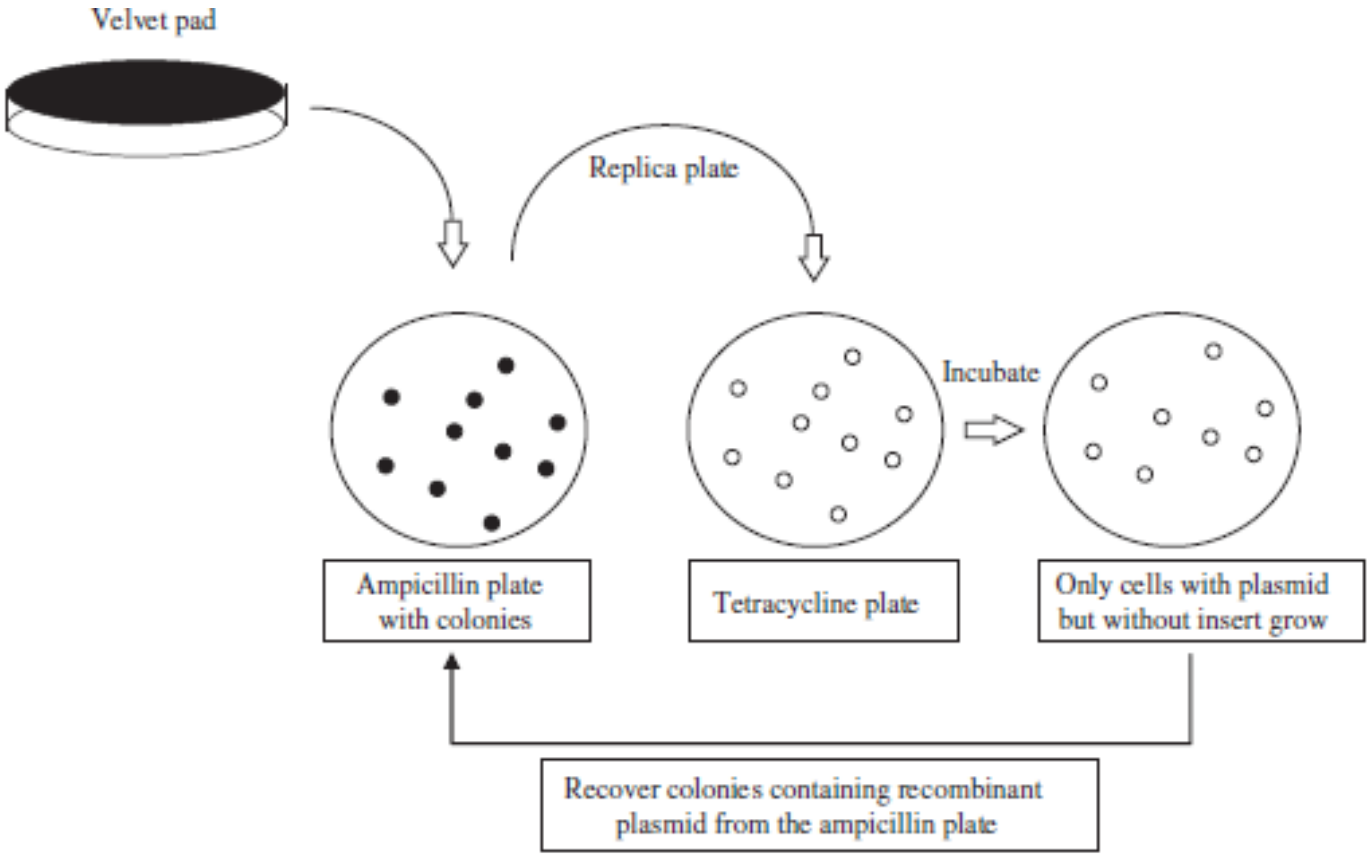

Velvet pad
Replica plate
Incubate
Ampicillin plate with colonies
Tetracycline plate
Only cells with plasmid but without insert grow
Recover colonies containing recombinant plasmid from the ampicillin plate

Figure Replica plating to detect recombinant plasmids. A sterile velvet pad is pressed on to the surface of an agar plate, picking up some cells from each colony growing on that plate. The pad is then pressed on to a fresh agar plate, thus inoculating it with cells in a pattern identical with that of the original colonies. Clones of cells that fail to grow on the second plate (*e.g.* owing to the loss of antibiotic resistance) can be recovered from their corresponding colonies on the first plate.

- **G.R:** **T**he transformed bacteria **should not be plated onto the selective medium immediately** after transformation, but should be incubated in liquid medium in the absence of antibiotics for a short time.

- To give the plasmid enough time for **replication and expression** of the antibiotic resistance protein,

2- Blue/white or LAC selection:

- In this type, the plasmid should contain the gene **(lacZ) that** encodes β-galactosidase.
- β-galactosidase hydrolyses X-gal (a colourless sugary substance), leading to the precipitation of **a blue insoluble material**.
- E. coli, lacZ gene is 'switched on' by adding the inducer **IPTG** b-galactosidase is produced.
- However, disruption of the gene by the insertion of DNA encoding the protein of interest means that X-gal is not hydrolysed.
- This means that a **host cell having a recombinant plasmid will be white or colourless.**
- whereas a host cell having a non-recombinant plasmid will be **blue.**

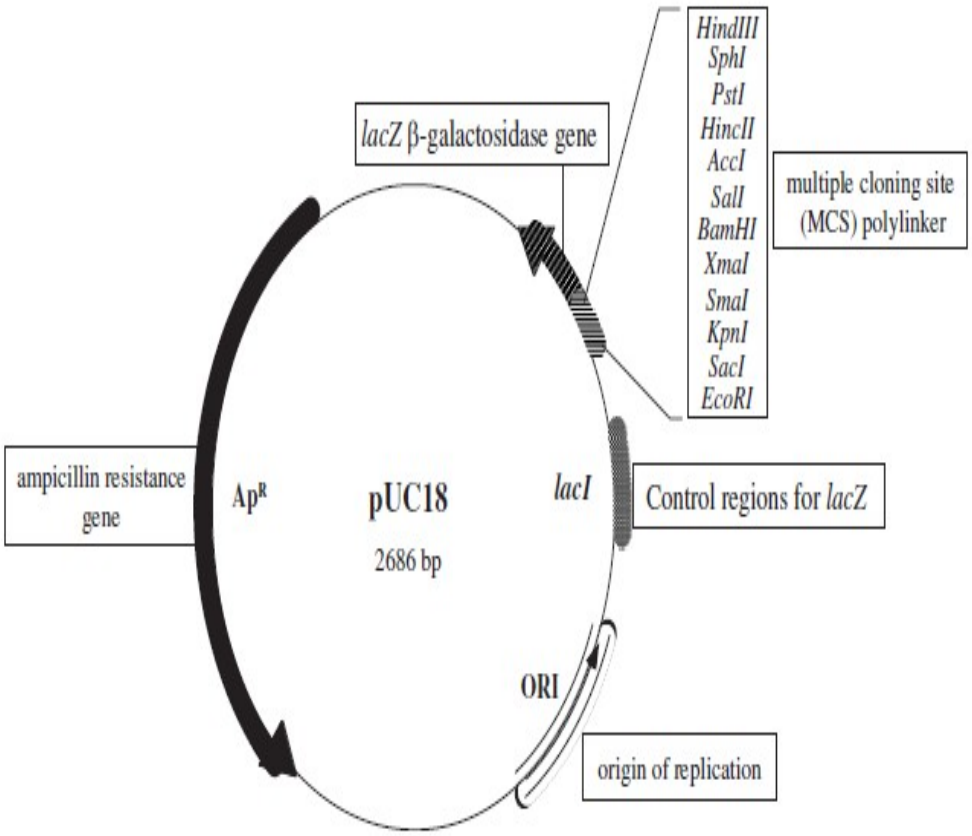


Figure Map and important features of pUC18, including restriction sites.

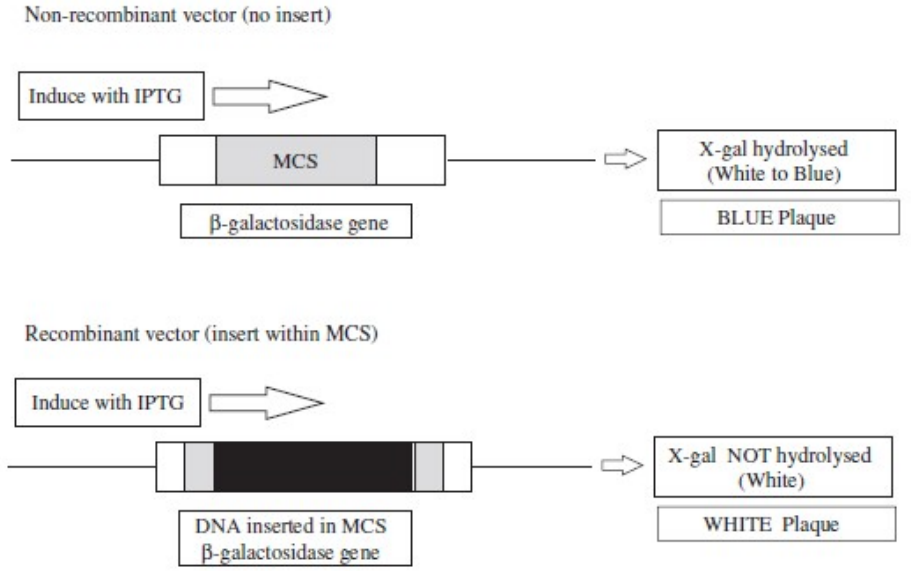
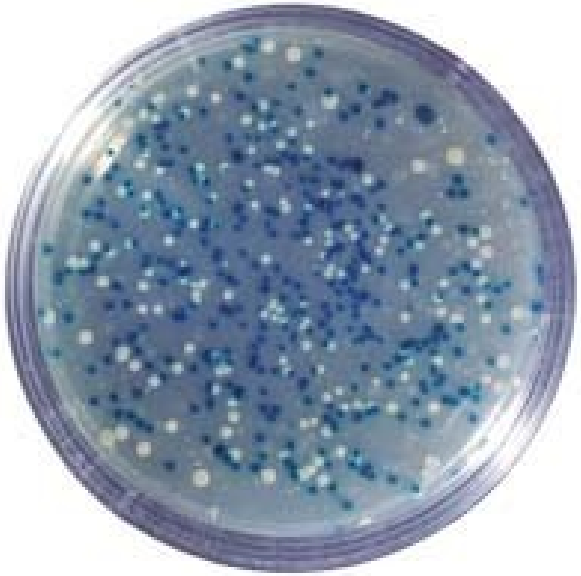


Figure 2.4 Principle of blue/white selection for the detection of recombinant vectors.



8- Plasmid extraction from bacteria (Minipreps or maxipreps):

1- Pick a colony from the agar plate (semi-solid)

2- grow colony in LB media (liquid)

3- Apply miniprep (or maxiprep) protocol to purify recombinant DNA from bacteria.

4- You can separate the target DNA from the plasmid using the former described methods (LEC.1).

5-Now, how can you separate your insert from the vector??

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THANK YOU!